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Dissertação
Mestrado em Biologia da Conservação

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Resumo

As invasões biológicas por espécies não-nativas constituem uma das principais ameaças aos ecossistemas naturais e à biodiversidade. Milhares de espécies foram extintas ou estão em risco devido a espécies introduzidas, em resultado de interações directas, competição ou transmissão de parasitas e agentes patogénicos. A maior parte das espécies consegue escapar dos seus parasitas quando são introduzidas em novos habitats, contudo alguns parasitas persistem nos seus hospedeiros e podem afectar seriamente as comunidades nativas. Várias têm sido as introduções de anfíbios por todo o mundo. A sua inconspicuidade e o facto de muitas vezes não afectarem directamente o Homem fazem com que pouca atenção lhes seja dada. Um anuro com grande difusão mundial é *Xenopus laevis*, sendo muitas vezes apontado como vector da quitridiomicose (doença resultante da infecção por *Batrachochytrium dendrobatidis*), e hospedeiro de parasitas originários do continente africano. A sua parasitofauna nativa caracteriza-se pela extraordinária riqueza, incluindo mais de 25 géneros de 7 grandes grupos de invertebrados. Já foi documentada a presença de alguns dos seus parasitas nativos em populações introduzidas, assim como de parasitas adquiridos nos novos habitats.

Com a descoberta de *X. laevis* em duas ribeiras em Oeiras (Portugal), tornou-se importante a realização de um estudo que caracterizasse a sua parasitofauna e possíveis impactos nas espécies nativas, neste caso a rã-verde *Pelophylax perezi*.

A amostragem decorreu durante o Verão. Foram procurados e capturados *X. laevis* e *Pelophylax perezi* com pesca eléctrica, tendo depois alguns sido aleatoriamente selecionados para dissecação (80 *X. laevis* e 18 *P. perezi*). Antes da dissecação, cada animal foi anestesiado numa solução de MS222 (0,1%) durante 15–30 minutos, seguindo a sua pesagem e medição (comprimento focinho-uróstilo - SUL). O sexo foi determinado pela observação directa das gónadas. Posteriormente, vários órgãos e tecidos foram removidos e examinados. Os macroparasitas encontrados foram medidos e identificados com recurso a bibliografia e à opinião de um especialista.

Foram encontradas três espécies de helmintes (*Protopolystoma xenopodis, Opisthodiscus cf. nigrivasis* e uma espécie não identificada) em *Xenopus laevis*, e cinco em *Pelophylax perezi* (*Opisthodiscus cf. nigrivasis, Sonsinotrema tacapense, Rhabdias bufonis* e 2 espécies não identificadas).

Protopolystoma xenopodis, a única espécie de parasita característica de *X. laevis* presente na população invasora, foi encontrada com uma prevalência de 55% e uma intensidade média de 2,59 parasitas adultos por hospedeiro. Valores tão elevados

poderão ter resultado de um confinamento de uma grande quantidade de *X. laevis* em corpos de água com caudal reduzido, facilitando altos níveis de invasão por *P. xenopodis* durante as épocas mais quentes e secas do ano. Houve uma relação negativa entre as dimensões de *P. xenopodis* e o decorrer dos meses de amostragem (r=-0,44, P<0,05), surgindo indivíduos mais pequenos em Agosto do que em Julho, o que indicia o aparecimento de parasitas jovens, recém-migrados dos rins de *X. laevis* à medida que o Verão avança. O sexo do hospedeiro não parece ser um factor determinante na 'primeira abordagem' deste parasita, tendo machos e fêmeas apresentado semelhantes taxas de infecção (χ^2=2,423, df=3, P=0,489), bem como semelhantes cargas parasitárias quando infectados (t_{42}=-0,609, P>0,05). Da mesma forma, o SUL de *X. laevis* parece não ter nenhuma relação com o número destes parasitas (machos: r=0,07, P>0,05; fêmeas: r=0,04, P>0,05). Contudo, existiram diferenças nas dimensões de *P. xenopodis* entre machos e fêmeas (t_{92}=2,271, P<0,05), tendo-se verificado as maiores diferenças em Agosto (t_{57}=2,227, P<0,05). Verificou-se uma redução nas dimensões de *P. xenopodis* à medida que o SUL dos machos de *X. laevis* aumentava (r=-0,284, P<0,05). Por outro lado, a dimensão destes helmintes aumentou proporcionalmente ao SUL das fêmeas de *X. laevis* (r=0,336, P<0,05), indicando uma maior probabilidade de novas infecções em fêmeas *X. laevis* mais novas e relações parasita-hospedeiro mais estáveis e duradouras em *X. laevis* mais velhas.

Foi também encontrado uma espécie de paranfistomatídeo, *Opisthodiscus cf. nigrivasis* em *X. laevis*, com uma prevalência de 33% e uma intensidade média de 2,23 parasitas por hospedeiro. Não existiu variação no número (t_{24}=0,582, P>0,05) nem nas dimensões (t_{49}=1,177, P>0,05) de *O. cf. nigrivasis* ao longo dos meses, apontando para uma não sincronização entre os ciclos de vida de parasita e anfíbio. Assim como em *P. xenopodis*, machos e fêmeas não apresentaram diferenças ao nível da taxa de infecção por *O. cf. nigrivasis* (χ^2=4,413, df=2, P=0,111) nem do número de parasitas por indivíduo infectado (t_{24}=-0,059: P>0,05). O sexo do hospedeiro também não pareceu influenciar as dimensões destes parasitas (t_{50}=-0,415, P>0,05). O tamanho das rãs não desempenhou um factor determinante no número de *O. cf. nigrivasis* que parasitam *X. laevis* (machos: r=-0,004, P>0,05; fêmeas: r=0,05, P>0,05); contudo foi observada uma correlação positiva entre o SUL das fêmeas de *X. laevis* e o comprimento *de O. cf. nigrivasis* (r=0,417, P<0,05). Nos machos não existiu qualquer relação (r=-0,051, P>0,05), sugerindo que estes parasitas encontrem condições de vida mais favoráveis e/ou uma mais fácil adaptação em fêmeas mais velhas, e por isso maiores.

Considerando todos os helmintes, 69% dos *Xenopus laevis* amostrados estavam infectados com pelo menos 1 indivíduo, com uma intensidade média de 3,25 parasitas por hospedeiro, num total de 179 parasitas. Não existiu diferenças entre o número de parasitas encontrados ao longo dos meses de amostragem (t_{50}=-0,855: *P*>0,05); contudo em Agosto houve níveis de infecção ligeiramente superiores, possivelmente devido ao aumento de parasitas recém-migrados de, pelo menos, uma das espécies. Também as taxas de infecção (χ^2=2,258, *df*=3, *P*=0,521) e o número de parasitas não variaram com o sexo (t_{53}=-0,130: *P*>0,05) e o SUL dos hospedeiros (machos: *r*=0,17, *P*>0,05; fêmeas: *r*=0,06, *P*>0,05).

Em *Pelophylax perezi*, todos os 18 indivíduos estavam infectados (prevalência de 100%), com uma média de 25 parasitas por hospedeiro, num total de 452 parasitas. O sexo do hospedeiro pareceu não exercer qualquer influência na biologia dos parasitas, sendo que as taxas de infecção foram semelhantes (χ^2=1,862, *df*=3, *P*=0,602), bem como o número total de parasitas entre machos e fêmeas (t_{15}=0,568: *P*>0,05).

A espécie nativa, *Pelophylax perezi*, é naturalmente mais parasitada (maior número de parasitas de um maior número de espécies) que *Xenopus laevis*, seja pelo longo período de coexistência nestas ribeiras, seja pela ausência em Portugal de várias espécies de parasitas típicas de *X. laevis*. A única espécie de parasita exótica que se conseguiu estabelecer em Portugal, juntamente com *X. laevis*, foi *P. xenopodis*, e a sua estrita especificidade parasita-hospedeiro minimiza a possibilidade de infecção da rã nativa. Assim, não se verificou indícios de transmissão de espécies de parasitas originárias de África para a única espécie de anfíbio cujo habitat se sobrepõe com a área de distribuição de *X. laevis* em Portugal. Contudo, ocorreu um padrão inverso de infecção, tendo existido a transmissão de parasitas de *P. perezi* para *X. laevis*. As outras 2 espécies encontradas em *X. laevis* terão sido adquiridas posteriormente à introdução no novo habitat. Comuns em *P. perezi*, *Opisthodiscus cf. nigrivasis* ocorreram em 50% das rãs-verdes amostradas, e na sua maioria bem desenvolvidos. O mesmo não aconteceu em *X. laevis*, em que estes helmintes estiveram presentes mas em número reduzido e em estádios menos desenvolvidos. No rectum de alguns indivíduos foram encontradas o que aparentou ser metacercárias enquistadas, sugerindo que *X. laevis* pode estar a ser parasitado através da ingestão de estádios larvares deste parasitas ou de um hospedeiro intermédio portador destes. Mesmo sem os níveis de sucesso com que parasita *P. perezi*, *O. cf. nigrivasis* parece estar a usar *X. laevis* como vector ou hospedeiro. Dado que esta espécie invasora é bastante abundante em certas zonas das

ribeiras, suplantando largamente os números de *P. perezi*, a população de *O. cf. nigrivasis* pode actualmente depender mais de *X. laevis* que de *P. perezi*.

Xenopus laevis é um forte competidor e um predador voraz. Para além dos impactos já conhecidos, temia-se que pudesse ser portadora de parasitas originários de África, capazes de pôr em causa a actividade e/ou a sobrevivência das espécies nativas, perturbando ainda mais o equilíbrio do ecossistema. Contudo, isto não foi verificado, tendo sido esta espécie invasora a ser infectada por parasitas autóctones. Ainda assim, a carga parasitária que apresenta não é tão elevada nem variada como a de espécies com que co-habita, ou tão alta como nos habitats onde é nativa, o que pode tornar esta espécie mais apta a dominar os ambientes onde foi recentemente introduzida.

Palavras-chave: Espécies exóticas invasoras, helminte, parasita, *Protopolystoma xenopodis*, taxa de infecção.

Abstract

Biological invasions by non-native species constitute one of the leading threats to natural ecosystems and biodiversity. Most of the animals can escape from its parasites when they are introduced into new habitats, however some persist in their hosts and may seriously affect the native communities. *Xenopus laevis*, an anuran with large world diffusion, is often a carrier of parasites originating from the African continent. With the recent discovery of *X. laevis* in two streams in Oeiras (Portugal), it became important to characterize its parasite fauna and its possible impacts on native species. In this study, we searched for macroparasites in 80 *X. laevis* and 18 native *P. perezi* living in the same stream sections. We found 3 species of helminths in *X. laevis*: *Protopolystoma xenopodis*, *Opisthodiscus cf. nigrivasis* and one unidentified species. *Protopolystoma xenopodis* had a prevalence of 55%, with an average of 2,59 parasites per infected host. *Opisthodiscus cf. nigrivasis* had a prevalence of 33% and a mean intensity of 2,23 parasites per host. *P. perezi* was found to be parasitized by 5 helminths: *Opisthodiscus cf. nigrivasis*, *Sonsinotrema tacapense*, *Rhabdias bufonis* and two unidentified species. *O. cf. nigrivasis* was the only species shared between the two hosts, and had a higher prevalence (50%) and a higher mean intensity (4,67 parasites per host) in *P. perezi*.

Considering all the helminths, 69% of the sampled *X. laevis* were infected with at least one species, with a mean intensity of 3,25 parasites per host. On the other hand, all the 18 individuals of *P. perezi* were infected with an average of 25 parasites per host.

In Portugal, *X. laevis* was the species that was found to be infected by autochthonous parasites, probably proceeding from *P. perezi*. Still, the parasite burden was not as high as in the species they co-exist with, or as high as in the habitats where it is native, which could enable this species to dominate the streams where it was recently introduced.

Keywords: Helminth, infection rate, invasive alien species, parasite, *Protopolystoma xenopodis*.

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INTRODUCTION

Biological invasions by non-native species constitute one of the leading threats to natural ecosystems and biodiversity (Wittenberg & Cock, 2001), and may soon surpass habitat loss as the main cause of environmental degradation globally (Chapin *et al.*, 2000).

The introduction of species outside their natural habitat increased dramatically due to human action, namely the globalization of trade, travel and transports (Genovesi & Shine, 2003). These activities, either intentionally or inadvertently, facilitate the movement of species through biogeographical barriers that would usually block their way (Genovesi & Shine, 2003). Today, invasive alien species (IAS) are one of the most significant drivers of environmental change worldwide (Sala *et al.*, 2000) and the ways in which they affect native species and ecosystems are varied and often subtle but irreversible (Wittenberg & Cock, 2001). IAS are associated to the extinction of various species (Wilcove *et al.*, 1998), degradation of aquatic and terrestrial environments (D'Antonio & Kark, 2002) and alteration of hydrology and nutrient cycles (Wittenberg & Cock, 2001), as well as economic and public health impacts (Genovesi & Shine, 2003). More specifically, hundreds of species have become extinct or are endangered due to introduced species, because IAS can prey on natives, compete with them, hybridize with them, attack them or even be vectors of pathogens (Wittenberg & Cock, 2001) and parasites.

Many biotic factors affect, independently or in interaction, the success of an invasive species. These include parasites and other pathogens which have high importance in the hosts' ecology (Gilbert, 2002). According to Torchin *et al*. (2003) the majority of animals may escape from their parasites and pathogens when introduced into new habitats. However, some parasites and/or pathogens can persist in their hosts and have serious impacts on native communities (Dove, 2000; Dobson & Hudson, 1986). This was the case for the parasite of avian malaria, *Plasmodium relictum*, which was introduced in the archipelago of Hawaii and allowed the invasive Eurasian birds, resistant to this illness, to prevail over the native birds, which were highly susceptible to the parasite (van Riper *et al.*, 1986). It is also the case for *Batrachochytrium dendrobatidis*, a pathogenic fungus that infects keratinized tissues of adult amphibians as well as of tadpoles (Longcore *et al.*, 1999; Garner *et al.*, 2005). This fungus is carried

by relatively resistant invasive species (e.g. *Lithobates catesbeianus, Eleutherodactylus coqui, Xenopus laevis*), being one of the greatest threats to the survival of amphibians worldwide (e.g. Beard & O'Neill, 2005, Longcore *et al.,* 1999; Garner *et al.,* 2005; Rachowicz & Briggs, 2007).

Several amphibian introductions have occurred all around the world (Pitt *et al.,* 2005). Their inconspicuousness and the fact that most of the time amphibians do not directly affect humans make them often go unnoticed until it is too late. The high reproductive capacity, high population density and generalist feeding habits characterize much of the invasive herpetofauna, making them difficult to eradicate (Pitt *et al.,* 2005; Kraus, 2007).

Some species of invasive amphibians are known due to their impacts. *Eleutherodactylus coqui* was accidentally introduced in Hawaii with imported plants from Puerto Rico (Kraus *et al.,* 1999 *in* Pitt *et al.,* 2005), during 1988-1995. Populations of this species were found on several islands, causing high levels of noise pollution through their callings, and impacts on populations of insects and native birds and bats (Pitt *et al.,* 2005; Beard & Pitt, 2005). Because of the large population densities and the late start of an eradication plan, their extermination was not possible. Also, the cane toad (*Rhinella marina*) is an amphibian invader that was introduced worldwide (Lever, 2001 *in* Shine, 2010), particularly in Australia, to control insect pests in sugar cane plantations (McKeown, 1978 *in* Pitt *et al.,* 2005; Shine, 2010). However, the toad not only was not effective in controlling these insects in Northern Australia, it became a plague that proliferated to other Australian regions (Seabrook, 1991; Phillips *et al.,* 2007; Shine, 2010). Predation, competition and toxic secretions produced by this toad led to the decline of several native species of reptiles and amphibians, as well as of reptile and mammalian predators (Seabrook, 1991; Shine, 2010).

Portugal is also affected by the introduction of exotic species. According to previous studies (Rebelo *et al.,* 2010), there are two populations of the African clawed frog, *Xenopus laevis* (Daudin, 1802), established in two streams (Lage and Barcarena), in Oeiras county, about 30 km west of Lisbon.

XENOPUS LAEVIS

The African clawed frog is an amphibian of the Order Anura and the family Pipidae, endemic in sub-Saharan Africa. It naturally occurs in almost all types of freshwater bodies of temperate, subtropical and tropical Africa, between Nigeria and South Africa (Tinsley *et al.*, 1996), but the subspecies *Xenopus laevis laevis* is confined to temperate zones, especially in the Cape region, in southwestern South Africa (Evans *et al.*, 2011; Lillo *et al.*, 2013).

Females of *X. l. laevis* are considerably larger than males and can measure up to 130 mm in length, while males are 10–30% smaller (Tinsley *et al.*, 1996). They have a very smooth and slimy skin with an olive-brown color with darker spots on the dorsum and a whitish ventral region.

Xenopus laevis is characterized by the presence of claws on three outer toes of the hind limbs (Channing, 2001). Like other pipids, it has no tongue or teeth (Stebbins, 2003) and it also shows specific adaptations to aquatic life, including retention of the lateral line system in adults (Munoz *et al.*, 2004), aquatic chemoreceptors (Elepfandt, 1996a, 1996b; Elepfandt *et al.*, 2000) and a body structure particularly adapted to swimming (Videler & Jorna, 1985). However, it can perform relatively long overland migrations (Measey & Tinsley, 1998; Eggert & Fouquet, 2006).

Its lack of tongue allows inertial suction feeding (Carreno & Nishikawa, 2010) of a wide variety of prey such as zooplankton, aquatic macroinvertebrates (Measey, 1998a), small fish and amphibians (Lafferty & Page, 1997) and even tadpoles and juveniles of its own species (Tinsley *et al.*, 1996). Individuals are also able to lunge out of the water to retrieve terrestrial prey, which are a frequent component of their diet (Measey, 1998b).

Xenopus laevis is ecologically distinguished from other species of amphibians by its capacity to survive for long dry periods (Alexander & Bellerby, 1938; Balinsky *et al.*, 1967; Lobos & Jaksic, 2005), or up to 12 months without feeding (Hewitt & Power, 1913; Tinsley *et al.*, 1996), and to show a high tolerance to salt water, as well as to anoxic conditions (Jokumsen & Weber, 1980). Adults and larvae perform well over a wide range of temperatures, and the tadpoles can also metamorphose under a large temperature range (Balinsky, 1981; Miller, 1982; Walsh *et al.*, 2008). It is a nocturnal species, with a high longevity in fully aquatic environments (15 years in nature: Flower, 1936; 30 years in captivity: Channing, 2001) and an extended breeding season, which

facilitates its high fertility. The aforementioned morphological, physiological and behavioral characteristics make *X. laevis* one of the most successful anurans in the laboratory and in nature (Measey *et al.*, 2012)

After the American bullfrog (*Lithobates catesbeianus*) and cane toad (*Rhinella marina*), *X. laevis* is probably the invasive amphibian species with the greatest worldwide diffusion (Lillo *et al.*, 2011). Its spread began in 1930's with the importation of these anurans to laboratories, where they were used as test animals in the diagnosis of human pregnancy, as well as a model species for developmental biology studies (Gurdon, 1996; Keller & Lodge, 2007; Weldon *et al.*, 2007). The necessity of breeding live animals has led indirectly to the spread of invasive populations (Fouquet & Measey, 2006).

The current distribution of *X. laevis* includes four of the five Mediterranean climate regions of the world, including its native region of South Africa, California (Crayon, 2005), Chile (Lobos & Measey, 2002) and the Mediterranean itself, with multiple introductions in Portugal (Rebelo *et al.*, 2010), Spain (Pascual *et al.*, 2007), France (Fouquet, 2001) and Sicily (Lillo *et al.*, 2005). Furthermore, it was introduced in the United Kingdom (Tinsley *et al.*, 1996; Measey & Tinsley, 1998), Germany, Netherlands, Ascension Island (Tinsley & McCoid, 1996), 12 states in the USA (Crayon, 2005; Krysko *et al.*, 2011; United States Geological Survey, 2011), Venezuela (Royero & Hernandez, 1996), Israel (Hatzofe, 2006) and Japan (Kobayashi & Hasegawa, 2005; Mitsuoka *et al.*, 2011).

Apart from predation impacts, there are indirect effects such as increased water turbidity and release of nutrients derived from disturbing the sediment, which can change the dynamics of the aquatic ecosystems (Lobos & Measey, 2002). Futhermore, introduced populations of *X. laevis* are often implicated as vector of chytridiomycosis (e.g. Solis *et al.*, 2011), a disease caused by the fungus *Batrachochytrium dendrobatidis*, which is lethal to many amphibians outside the African continent.

Perhaps due to its wide use in laboratory and large spread as an invasive species, *X. laevis* is one of the best studied species in terms of its parasite fauna, characterized by an extraordinary richness, including over 25 genera of 7 major groups of invertebrates (Tinsley, 1996), (Table 1).

Many of these parasites are highly specialized and possess unique adaptations, having a dual origin: one part of the parasite fauna of *X. laevis* has affinity with other groups represented in frogs, while the other part is related to the most common parasites in fish (Tinsley, 1996). Thus, as a group, this parasite fauna stands apart from almost all its respective relatives, with the vast majority specific to *Xenopus* (Tinsley, 1996).

Table 1. The parasite fauna of *Xenopus laevis* (adapted from Tinsley, 1996).

Parasite	Infection Site
MONOGENEA	
Protopolystoma xenopodis	Urinary bladder, kidneys
Gyrdicotylus gallieni	Mouth
DIGENEA	
Neascus sp.	Lateral line
Echinostomum xenopodis	Eyelids
Clinostomum sp.	Body cavity
Cercaria xenopodis	Lateral line
Opisthioglyphe xenopodis	Dermis
Dollfuschella rodhaini	Stomach
Oligolecithus elianae	Intestine
Xenopodistomum xenopodis	Gall bladder
Progonimodiscus doyeri	Rectum
Diplostomum (Tylodelphys) xenopodis	Pericardium
CESTODA	
Cephalochlamys namaquensis	Intestine
NEMATODA	
Camallanus kaapstaadi	Oesophagus
Camallanus xenopodis	Intestine
Batrachocamallanus slomei	Stomach
Pseudocapillaroides xenopodis	Epidermis
Microfilariae	Blood
ACARI	
Xenopacarus africanus	Nostrils, eustachian passages
HIRUDINEA	
Marsupiobdella africana	External skin

A part of the parasite fauna of *X. laevis* has affinities with that represented in other anuran amphibians. Thus, *Protopolystoma* is related to the genus *Polystoma* which occurs in the urinary bladders of a wide range of anurans. *Progonimodiscus* is equivalent to *Diplodiscus*, a paramphistome, which occurs in the corresponding site – the rectum – in other frogs and toads. These links reflect the common ancestry of the

parasites which probably infected the early anuran stock and then evolved with their respective host groups (Tinsley, 1996).

Almost all organ systems of *Xenopus* provide habitats for parasites, especially those which are in direct communication with the outside, like the alimentary canal and linked organs without any physical barrier to entry. Regions including pericardial and peritoneal cavities, lateral lines system, eyelids and general musculature, harbour parasites that use *Xenopus* as an intermediate host, since there is no direct exit from those sites (Tinsley, 1996). The external skin surface is generally not infected by permanent parasites (nor bacteria), possibly due to the periodic moulting, specific peptide skin secretions and/or the drying of the skin during overland migration/aestivation (Tinsley, 1996).

The life cycles of the 18 parasites associated with *Xenopus* represent a major part of the diversity shown by helminths. Some of the parasites have indirect life cycles and exploit *Xenopus* as an intermediate host, awaiting passage to final hosts which are predators of *Xenopus* (and usually also of fish) (Tinsley, 1996). These parasites may be pathogenic, infecting the heart, body musculature, eyes and lateral line system. By interfering with normal function, infection increases the risks of predation and thus facilitates completion of the parasite's life cycle (Tinsley, 1996). Other parasites, with direct life cycles, use *Xenopus* as a final host (in which their sexual reproduction occurs), and invade it by a variety of routes (Tinsley, 1996). For most of these parasites, pathogenic effects are difficult to detect and infection levels tend to occur below the point at which damage may be serious.

In the life-cycle patterns of most helminths, the principles of transmission are typical of parasites of fish. Transmission is water-borne across the spectrum of parasites. *Xenopus* may become infected in one of three ways:

a. active invasion, involving an infective stage which is specifically equipped for locomotion, host location and recognition, and for migration to the preferred site of infection within the host's body;

b. passive invasion, generally with ingested food items, and the parasite has no control over events during transmission except that emergence within the host's gut may be triggered by specific cues, followed by active migration to the final habitat;

c. host-to-host transfer, mediated by a vector, most commonly feeding on the blood of successive hosts and incidentally transferring parasites which may or may not require a period of development in the vector (Tinsley, 1996).

Although the existence of parasites in feral populations of *X. laevis* is already documented, little is known about its parasites outside Africa (Kuperman *et al.,* 2004). The African species with direct life cycles dominate the list of parasites carried to new ecosystems, while among the 13 African parasites with indirect life cycles only one managed to get a suitable intermediate host for its survival in California (Kuperman *et al.,* 2004). Thus, *Protopolystoma xenopodis* has been found in populations of *X. laevis* in Wales and California, and *Cephalochlamys namaquensis* found on the Isle of Wight (U.K.) (Lafferty & Page, 1997; Tinsley & Jackson, 1998; Jackson & Tinsley, 2001a, 2001b) and California (Kuperman *et al.,* 2004). Also *Gyrdicotylus gallieni* and the protozoans *Balantidium xenopodis* and *Protoopalina xenopodus*, originating from Africa, are present in feral populations of *X. laevis* in U.S.A. (Thurston, 1970; Tinsley, 1996; Kuperman *et al.,* 2004). A metacercaria of *Clinostomum* sp. was identified in Africa and California, though it is unknown whether it is the same species (Kuperman *et al.,* 2004). However, these parasites were not found in other anurans in North America (Ingles, 1936; Baker, 1987; Goldberg *et al.,* 1995; Goldberg *et al.,* 1996a; Goldberg *et al.,* 1996b; Goldberg *et al.,* 1998).

Likewise, it is possible for *X. laevis* to acquire new parasites in areas where it was introduced, which presumably are non-host specific to the animals they infect. Kuperman *et al.* (2004) documented the presence of parasites in *X. laevis*, especially of birds, using fish as an intermediate host, including *Acanthocephalus* sp. and the nematodes *Contracaecum* sp. and *Eustrongyloides* sp. This is most likely a result of fully aquatic life cycle of these frogs, which is closer to fish than the native semi-terrestrial amphibians.

PELOPHYLAX PEREZI

The Iberian green frog, *Pelophylax perezi* (Seoane, 1885), is an amphibian of the order Anura and family Ranidae endemic to the Iberian Peninsula and southern France. Its habitat includes streams, ponds, marshes, permanent and temporary lakes, dams, agricultural and urban areas (Bosch *et al.,* 2009 *in* IUCN, 2013; Ferrand de Almeida *et al.,* 2001). It is a mostly aquatic species, with preference for areas of calm, relatively

deep waters (Lizana *et al.,* 1987), and tolerant of habitat disturbance (Beja & Alcazar, 2003; Bosch *et al.,* 2009 *in* IUCN, 2013).

This frog has a robust body, which normally varies between 50 and 75 mm in length in adults, although some females are known to reach 100 mm (Curt & Galán, 1982 *in* Lizana *et al.,* 1987). The skin is smooth and slightly warty, with two dorsal-lateral folds. Its dorsal coloration is quite variable, generally being green, more or less dark, with black spots on most individuals, and a light green vertebral line that is not always present. The ventral region has a grayish white color. It presents robust forelimbs with four fingers, and long, strong and muscled hindlimbs, with five webbed toes (Ferrand de Almeida *et al.,* 2001).

Pelophylax perezi has more aquatic habits that the majority of anurans with which it co-occurs (Stebbins, 1966 *in* Lizana *et al.,* 1987) and its activity is both diurnal and nocturnal. The tadpoles are capable of growing in warmer waters than other species, a factor that allows this species to start its reproduction later than other Mediterranean anurans (Díaz-Paniagua, 1992).

The diet of adults is based on insects, spiders, worms, crustaceans, mollusks and even small fish and amphibians, including their own species. The tadpoles feed mainly on algae, detritus and phanerogams, typically inhabiting the bottom of water bodies (Díaz-Paniagua, 1985). The tadpoles develop mainly during late spring and summer, when resource availability is small, and thus their diet has low diversity (Díaz-Paniagua, 1985). Their high swimming ability and activity levels allow for a greater exploitation of food resources (Díaz-Paniagua *in* Egea-Serrano, 2006).

It is the most widespread and abundant species of the Portuguese amphibian fauna, and apparently is expanding its distribution to areas of higher altitude, possibly as a result of climate change (Bosch *et al.,* 2009 *in* IUCN, 2013).

Pelophylax perezi is the only amphibian in Portugal that co-exists with the invasive populations of *X. laevis*. It is not known if it was previously the only species existing in the invaded streams or if it is simply the only native amphibian that is able to coexist with *X. laevis*. In fact, the overlap of the preferential habitats of both species is very large.

Forty four species of helminths, including trematodes, nematodes and cestodes, were already found in this frog (Egea-Serrano, 2006; and references therein).

OBJECTIVES

The main goal of this work was to characterize the parasite fauna of *Xenopus laevis* in Oeiras' streams and examine the possibility of parasite exchange with the native species, *Pelophylax perezi*.

More specifically, we intended: **i**) to characterize the parasitological fauna of *X. laevis* and *P. perezi* over a breading season; **ii**) to characterize the infected subpopulation, by comparing the sex-ratio and the dimensions of infected vs non-infected individuals; **iii**) to identify the parasite species that might be using *X. laevis* as a vector to infect other amphibians; **iv)** to identify the native helminth parasites that naturally infect *P. perezi* and that might have invaded the *X. laevis* specimens.

In accordance with a major principle of ecological parasitology, a host species with a particular parasite fauna in its native range will lose a number of parasite species as a result of introduction in a new environment and will acquire additional non-host specific parasites in the new habitat (Dogiel, 1938 *in* Kuperman *et al.*, 2004; Kennedy & Bush, 1994; Torchin & Mitchell, 2004). By comparison with other invasive populations of *X. laevis*, we expected to find native parasites from South Africa in the specimens captured in the Oeiras' streams. We also followed up the possibility of finding parasites acquired from the native fauna at the introduction site (Torchin & Mitchell, 2004), as in the populations studied in California (Kuperman *et al.*, 2004).

MATERIALS AND METHODS

STUDY AREA

Fieldwork was conducted in Lage and Barcarena streams. These streams run in roughly parallel courses, with their sources east of Sintra Mountain and mouths in the Tejo Estuary, on the beaches of Santo Amaro and Caxias, respectively (Fig. 1; Annex I). Lage stream has a total length of 15,8 km, while the Barcarena stream is longer, covering a little more than 19 km. The minimum distance between the two streams is about 3,25 km (Fig. 1).

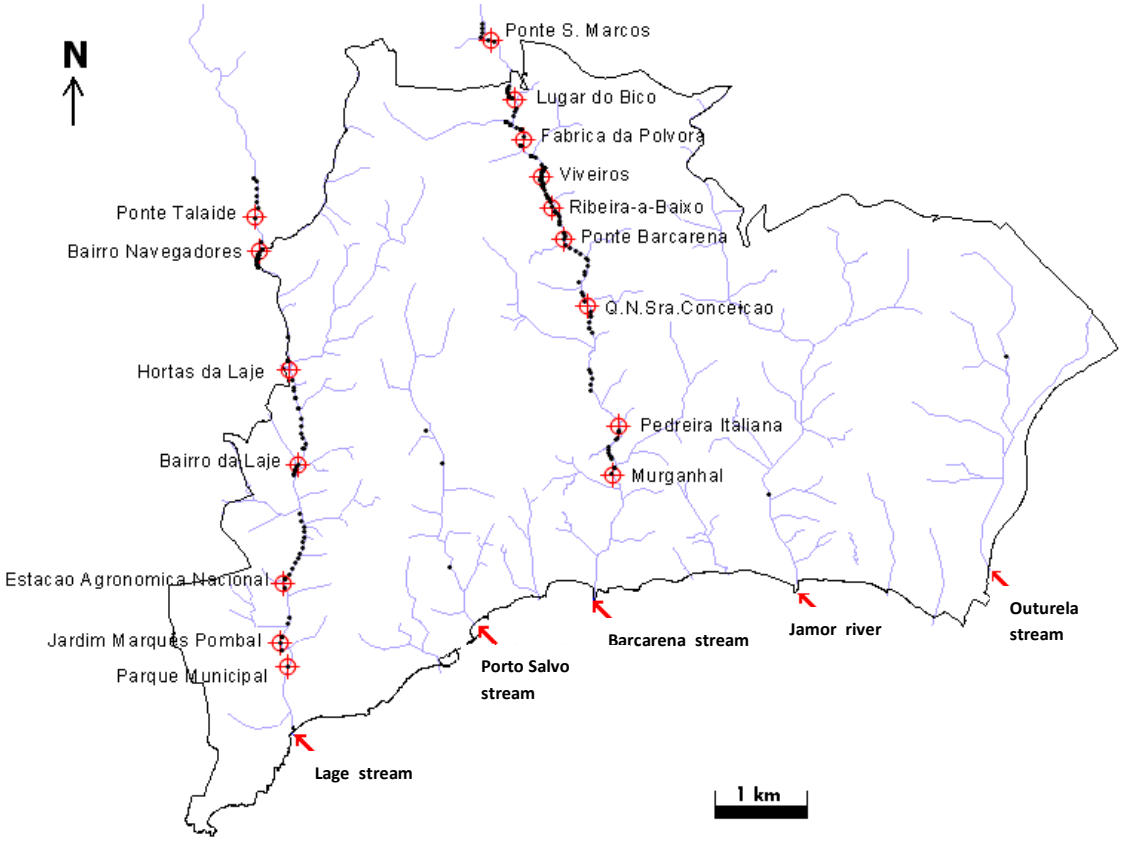

Fig. 1. Map of the study area, showing the streams of Oeiras and the sites where *Xenopus laevis* were searched for during the eradication plan and, consequently, the sampling period.

The basins of both streams are very small and isolated from other similar basins in western Portugal by hilly landscapes, except the portions that cross through Sintra municipality. In Oeiras municipality, the area surrounding the streams is urbanized and the banks were channeled with concrete walls.

The region is characterized by its Mediterranean climate, with an irregular water regime: there may be very strong stream flow, with floods during winter, but the stream dries almost completely in late summer.

After having identified the sites with still water during the summer, these were visited in order to sample *Xenopus laevis*. Samples were collected at 11 still water sites, all located within the Oeiras county: 2 in the Lage stream (Bairro dos Navegadores and Jardins do Marquês de Pombal) and 9 in the Barcarena stream (Ponte de S. Marcos, Lugar do Bico, Entre-Lugar-do-Bico-e-Fábrica-da-Pólvora, Fábrica da Pólvora, Tributário, Foz do Tributário, Viveiros, Ribeira-Abaixo and Murganhal), being the coordinates of these sites listed in Annex I.

METHODOLOGY

Fieldwork took place over five weeks, between 3–14 of June, 8–19 of July and 26–30 of August 2013. Thus, we focused the sampling period in the summer months, when the water level is low enough to allow access to the deepest reaches of the streams. One further sampling was conducted on October 16th. Additional fieldwork targeting to *Pelophylax perezi* took place between 22–25 of July 2014.

In every site visited adult *Xenopus laevis* were searched for and captured with an electrofishing set (SAMUS-752GN). Electrofishing was performed for periods of 45–60 minutes at a constant frequency of 30 Hz. All captured animals were brought to the laboratory, where they were kept for 1–4 days. Of those, a total of 80 individuals were randomly selected for dissection. The same methods were followed in order to capture *P. perezi* (18 individuals).

Before dissection, each animal was anaesthetized in a 0,1% MS222 solution (buffered with sodium bicarbonate) for 15–30 minutes, and its weight and body length (snout-urostyle length – SUL) were recorded. Sex was determined by direct observation of the gonads. Small animals with undifferentiated gonads were classified as juveniles.

During the dissection the following organs and tissues were removed and examined in Petri dishes submerged in physiological saline solution: stomach, intestine, rectum, urinary bladder, lungs, gall bladder, heart and oral cavity. All those organs and tissues were dissected under a microscope and examined for the presence of parasites. Kidneys were not examined for monogenean larvae because it is a time consuming process, requiring experience.

The parasites found were collected and fixed in 10% formalin under light coverslip pressure to prevent folding of the parasite's body. Subsequently they were photographed in a Leica DFC290 camera mounted on a Leica DM2000 stereo microscope. The parasites were identified based on expert opinion and on relevant bibliography (Martínez-Fernández *et al.*, 1988).

Body length and body width were measured for all parasites identified as *Protopolystoma xenopodis* and *Opisthodiscus cf. nigrivasis*. All measurements were taken with image analysis software, ImageJ, and were used to calculate the total surface area of the parasites, as in Tinsley *et al.* (2011). The number of inter-caecal anastomoses of *P. xenopodis* varies among populations (R. C. Tinsley, pers. comm.) and therefore were counted in each individual.

Standard parasitological parameters, such as prevalence (proportion of the population infected), abundance (mean number of parasites of both infected and uninfected *Xenopus*), and mean intensity (mean number of parasites of infected hosts) were determined for the infections of *X. laevis* and *P. perezi*.

By multiplying the prevalence of the two identified helminths that were found to parasitize *X. laevis*, we calculated the theoretical probability of a simultaneous infection with both these parasites, and compare it with the number of observed multiple infections to assess if infection by one parasite somehow facilitates (or hinders) infection by another.

Chi-square tests were used to compare infection rates between male and female *Xenopus*; t-tests were used to compare the number and size of parasites between genders and between sampling dates. Relationships between parasite dimensions and *X. laevis* dimensions, and between parasite dimensions and day of the year were tested using Pearson's correlations.

All tests were performed with STATISTICA 10 and Microsoft Excel.

RESULTS

LIST OF HELMINTHS FOUND

Three species of helminth parasites were found in the dissected animals: the monogenean *Protopolystoma xenopodis* (Price, 1943), from the family Polystomatidae; the digenean *Opisthodiscus cf. nigrivasis* (von Meheli, 1929), from the family Paramphistomidae; and one unidentified species (unidentified A), (Fig. 2).

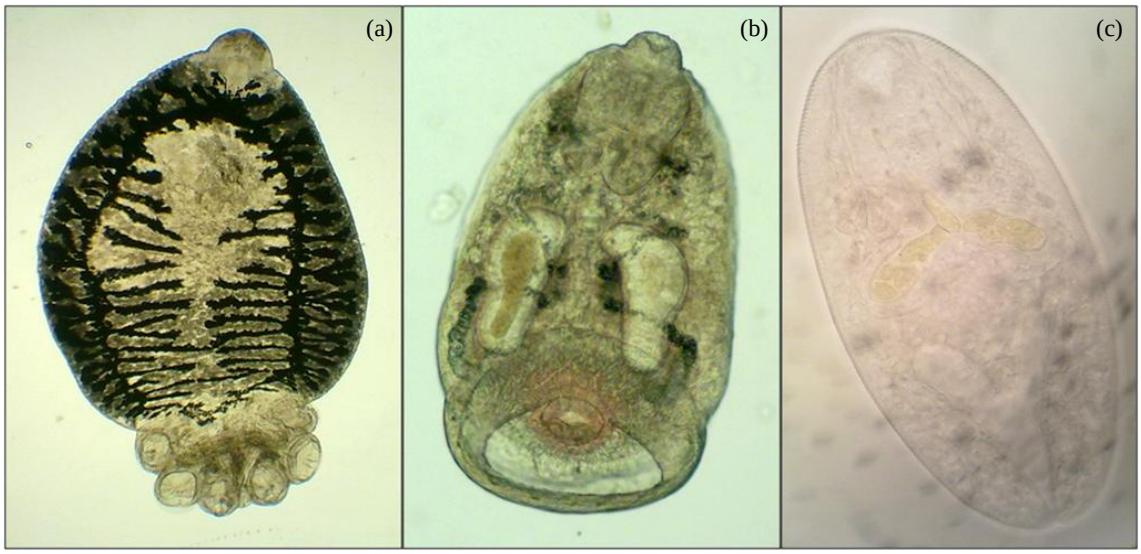

Fig. 2. Parasites found in *X. laevis*: (a) *Protopolystoma xenopodis*, (b) *Opisthodiscus cf. nigrivasis*, (c) unidentified trematode; type "A".

We found what possibly are five species of helminths in the dissected *Pelophylax perezi*: the digenean *O. cf. nigrivasis*, from the family Paramphistomidae; the digenean *Sonsinotrema tacapense* (Sonsino, 1894), from the family Lecithodendriidae; the nematode *Rhabdias bufonis* (Schrank, 1788), from the family Rhabdiasidae; and two unidentified species (unidentified B and C), infecting the intestine and the rectum (Fig. 3 and 4). Furthermore, two leeches (Annelida: Hirudinae) were found on one individual.

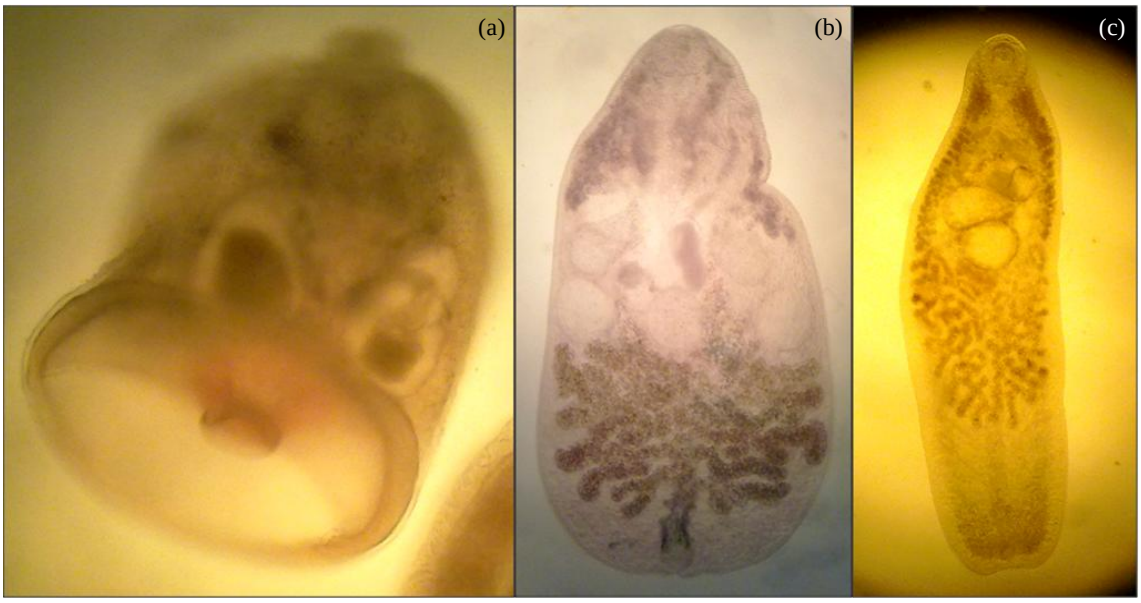

Fig. 3. Parasites found in *P. perezi*: (a) *Opisthodiscus cf. nigrivasis*, (b) *Sonsinotrema tacapense*, (c) unidentified trematode; type "B".

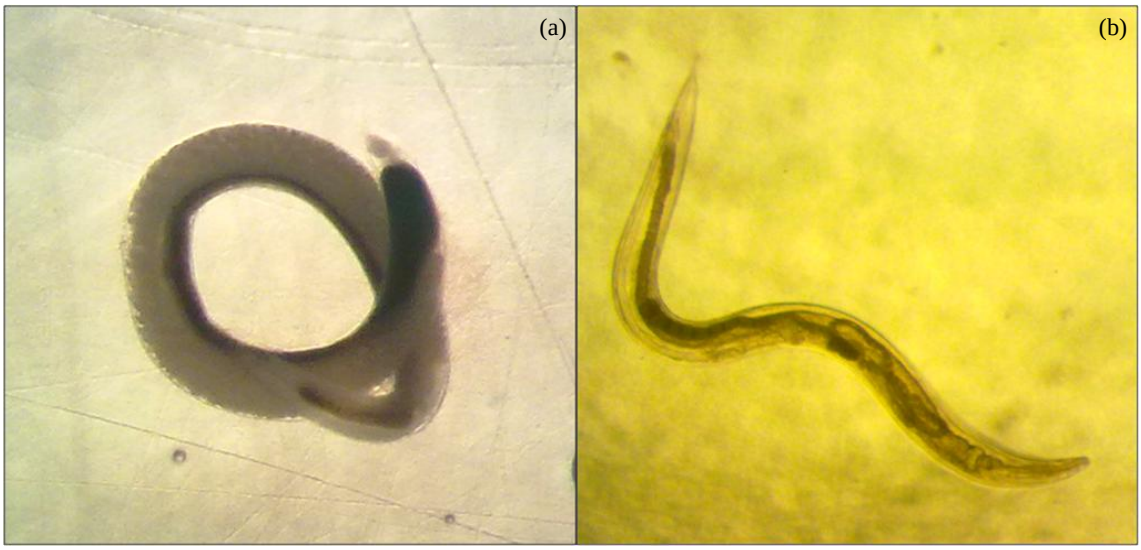

Fig. 4. Parasites found in *P. perezi*: (a) *Rhabdias bufonis*, (b) Unidentified nematode; type "C".

As far as we know, this is the first report of *O. cf. nigrivasis* infecting *X. laevis.* This is a species of parasite that occurs frequently in the only other anuran living in the sampled streams, *P. perezi*. The third species, of which seven specimens were found, remains unidentified.

Protopolystoma xenopodis

We found a total of 114 adult *P. xenopodis*, in the urinary bladders of the dissected *X. laevis*. The prevalence of *P. xenopodis* in our sample was 55%, with a mean intensity of 2,59 parasites per host (Table 2).

Table 2. Details of the *Protopolystoma xenopodis* infection in *Xenopus laevis* in Oeiras (Portugal) in 2013. Two of the sampled frogs were juveniles, so sex recognition was not possible.

	July			August			October			Total		
	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female
Number of *X. laevis*	30	18	12	40	21	19	10	4	4	80	43	35
Number of Infected *X. laevis*	17	11	6	25	11	14	2	1	1	44	23	21
Number of *P. xenopodis*	36	23	13	71	37	34	7	4	3	114	64	50
Prevalence	56,7	61,1	50	62,5	52,4	73,7	20	25	25	55	53	60
Abundance	1,2	1,3	1,1	1,8	1,8	1,8	0,7	1	0,8	1,4	1,5	1,4
Mean intensity	2,1	2,1	2,2	2,8	3,4	2,4	3,5	4	3	2,6	2,8	2,4

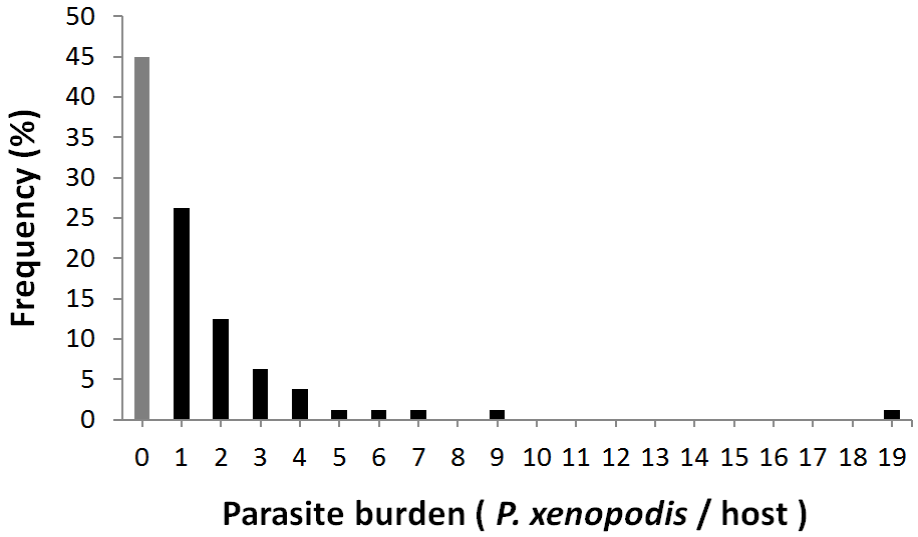

Fig. 5. Frequency distributions of *P. xenopodis* burden in *X. laevis*

Ninety three percent of the infection cases were at or below the documented common maximum (Tinsley, 1996) of six adult worms per host, but we also recorded exceptional cases of 7, 9 and 19 adults worms per host (Fig. 5).

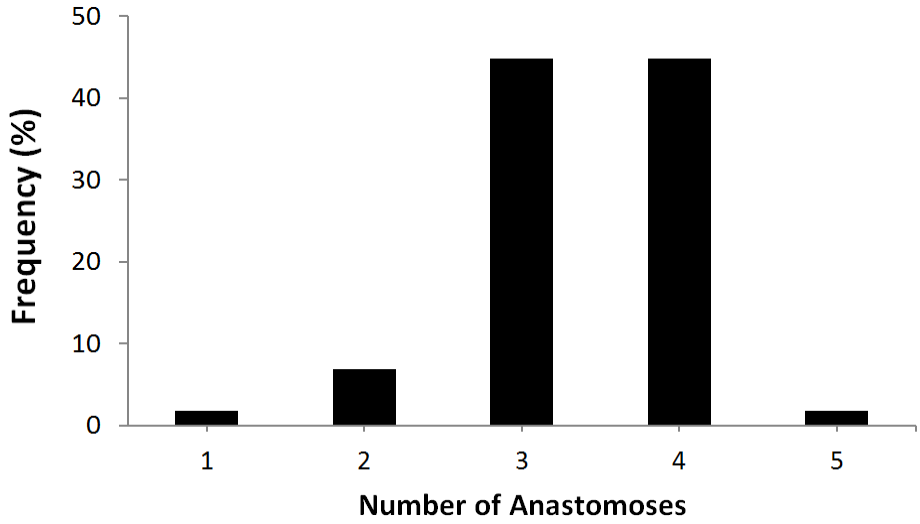

Fig. 6. Frequency distribution of the number of inter-caecal anastomoses of *P. xenopodis*.

Among the adult *P. xenopodis* of different sizes with perfectly visible post-ovarian median caecal branches, the majority of the individuals presented 3 (n=26; 44,8%) or 4 (n=26; 44,8%) inter-caecal anastomoses, with a few cases of individuals with 1 (1,7%), 2 (6,9%) and 5 (1,7%) anastomoses (Fig. 6).

Relations with sampling date

Prevalence was higher in July (57%) and in August (62,5%) than October (20%). However, the mean intensity reached values of 3,5 in October and only 2,12 and 2,84 in July and in August, respectively.

There were no differences between the number of *P. xenopodis* found in July and August (t_{39}=-0,084: P>0,05).

There were significant differences between the dimensions of *P. xenopodis* sampled in July and August (Area: t_{85}=4,943: P<0,05). The mean area in July (2,19 mm^2) was significantly higher than in August (0,97 mm^2). Accordingly, there was a negative correlation between "Length *P. xenopodis*" and "Day of the Year" (r=-0,44, P<0,05), as well as "Area *P. xenopodis*" and "Day of the Year" (r=-0,43, P<0,05), (Fig. 7).

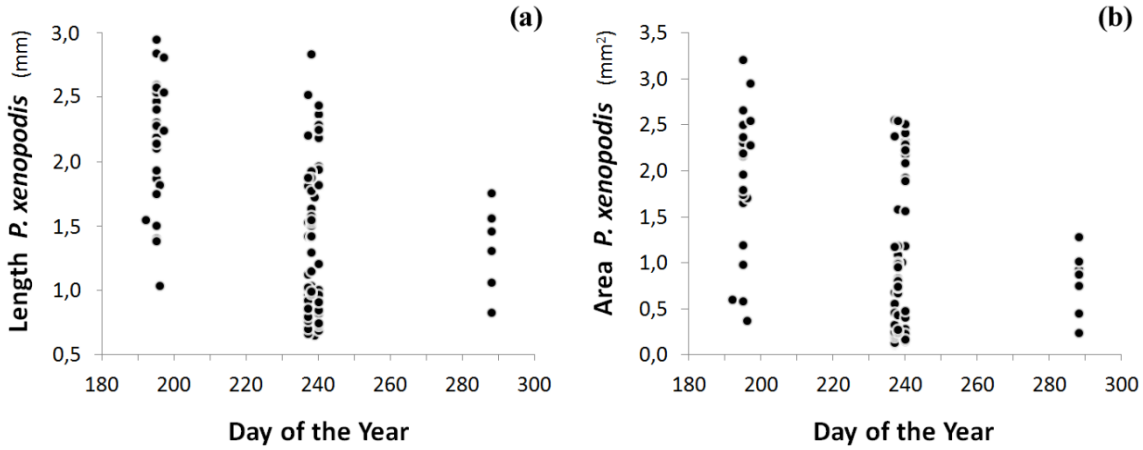

Fig. 7. Relation between: (a) the length of *P. xenopodis* and the day of the year; (b) the area of *P. xenopodis* and the day or the year.

Differences between sexes

Overall, the infection rate of males and females was similar (χ^2=2,423, *df*=3, *P*=0,489). There was also no difference between the parasite burden (number of *P. xenopodis* per individual) of males and females (t_{42}=-0,609: *P*>0,05). Infected males had an average of 2,05 parasites per individual against 2,38 in each female. There were no differences in the average number of *P. xenopodis* per individual between genders in July (t_{15}=-0,146: *P*>0,05) and in August (t_{22}=0,669: *P*>0,05).

There were significant differences between the dimensions of *P. xenopodis* parasiting male and female *Xenopus* (Area: t_{92}=2,271: *P*<0,05). The parasites found in males had a higher mean area (1,55 mm^2) than the parasites present in females (1,01 mm^2). However, that difference was not found when we analyzed only the individuals captured in July (n=28), (t_{26}=0,438: *P*>0,05). In August (n=59) there were differences in the dimensions of parasites between males and females (t_{57}=2,227: *P*<0,05). In that month, males had parasites with a mean total area of 1,21 mm^2 and females of 0,68 mm^2.

Relation with frog SUL

There was no association between the number of *P. xenopodis* and the SUL of both male and female *X. laevis* (Males: *r*=0,07, *P*>0,05; Females: *r*=0,04, *P*>0,05), (Fig. 8).

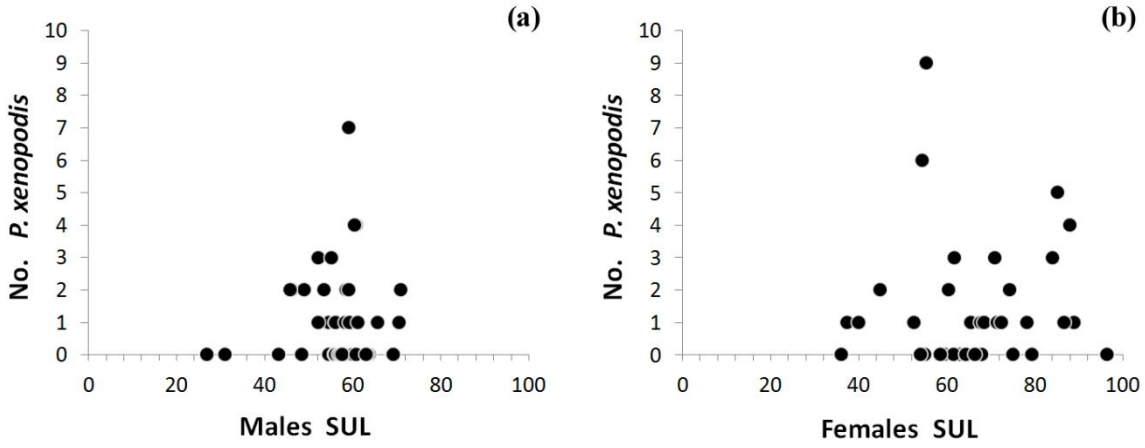

Fig. 8. Relation between: (a) the number of *P. xenopodis* and the SUL of male *X. laevis*; (b) the number of *P. xenopodis* and the SUL of female *X. laevis*.

However, analyzing the genders separately, there was a positive correlation between "Length *P. xenopodis*" and SUL of female *X. laevis* (r=0,336, P<0,05) and a negative relation between "Area *P. xenopodis*" and SUL of male *X. laevis* (r=-0,284, P<0,05). All other tests had non-significant results ("Length *P. xenopodis*" *vs* "Males' SUL": r=-0,206, P>0,05; "Area *P. xenopodis*" *vs* "Females' SUL": r=0,166, P>0,05), (Fig. 9).

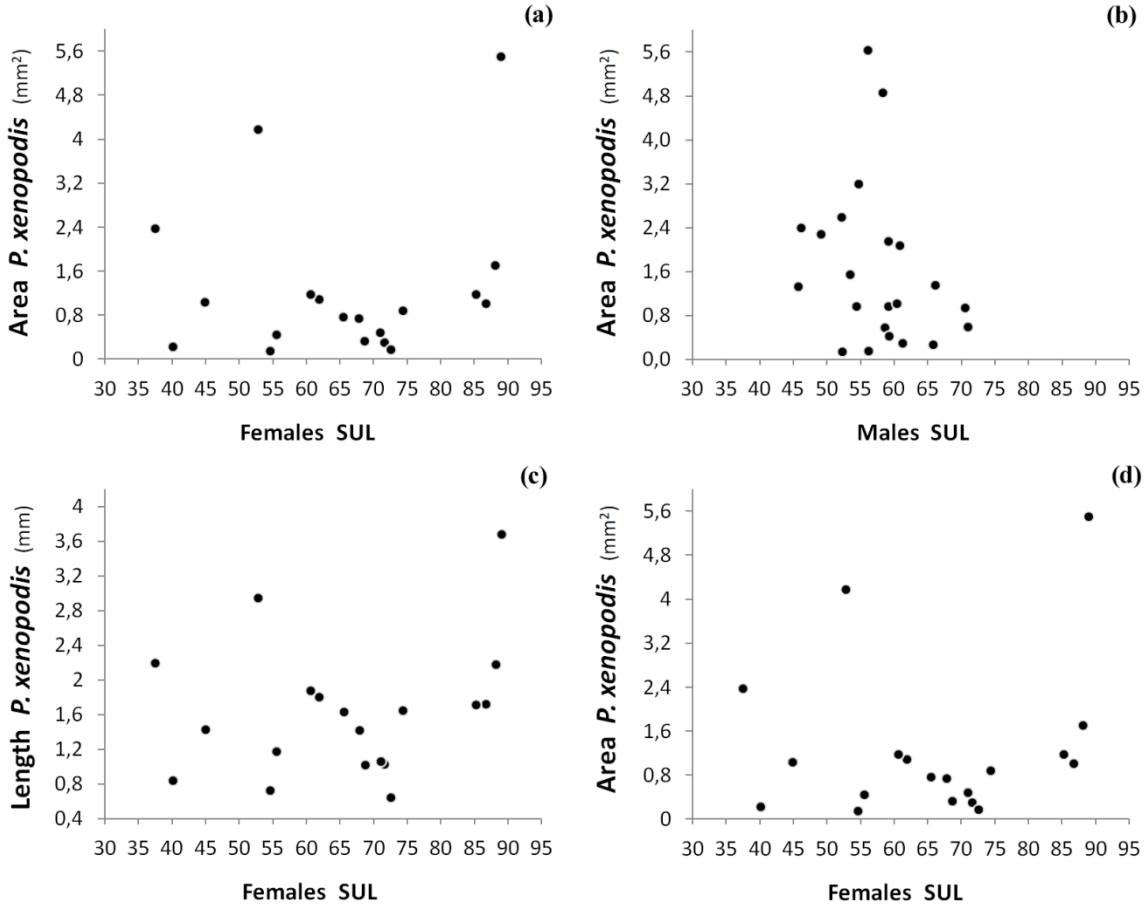

Fig. 9. Relation between: (a) the length of *P. xenopodis* and the SUL of male *X. laevis*; (b) the area of *P. xenopodis* and the SUL of male *X. laevis*; (c) the length of *P. xenopodis* and the SUL of female *X. laevis*; (d) the area of *P. xenopodis* and the SUL of female *X. laevis.*

Opisthodiscus cf. nigrivasis

We found 58 *O. cf. nigrivasis*, all located in the rectums of the dissected *X. laevis*. The prevalence of *O. cf. nigrivasis* in our sample was 33%, with a mean intensity of 2,23 parasites per host (Table 3).

Table 3. Details of the *O. cf. nigrivasis* infection in *Xenopus laevis* in Oeiras (Portugal) in 2013. Two of the sampled frogs were juveniles, so sex recognition was not possible.

	July			August			October			Total		
	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female
Number of *X. laevis*	30	18	12	40	21	19	10	4	4	80	43	35
Number of Infected *X. laevis*	6	3	3	19	7	12	1	0	1	26	10	16
Number of *O. cf. nigrivasis*	16	6	10	41	16	25	1	0	1	58	22	36
Prevalence	20	16,7	25	47,5	33,3	63,2	10	0	25	33	23	46
Abundance	0,5	0,3	0,8	1,0	0,8	1,3	0,1	0	0,3	0,7	0,5	1,0
Mean intensity	2,7	2	3,3	2,2	2,3	2,1	1	0	1	2,2	2,2	2,3

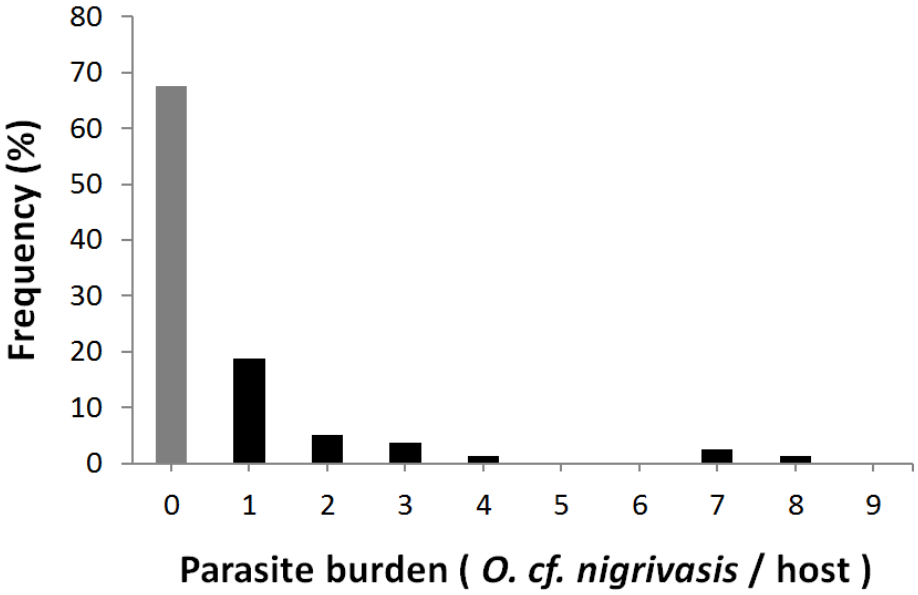

Fig. 10. Frequency distributions of *O. cf. nigrivasis* burden in *X. laevis*.

Almost 58% of the infection cases corresponded to *X. laevis* infected with only one *O. cf. nigrivasis*. The parasite burden ranged from 1–8 worms per host in the infected *X. laevis*, while 67% of the sampled population had none of these parasites (Fig. 10).

Relation with sampling date

The prevalence was higher in August (47,5%) than in July (20%) and October (10%). The mean intensity reached values of 2,67 in July and only 2,16 and 1 in August and in October, respectively.

There were no differences between the number of *O. cf. nigrivasis* found in July and August (t_{24}=0,582: *P*>0,05).

Also, there were no significant differences in the dimensions of *O. cf. nigrivasis* sampled in July and August (Length: t_{49}=1,268: *P*>0,05; Area: t_{49}=1,177: *P*>0,05). The mean area in July (0,287 mm^2) was slightly higher than in August (0,227 mm^2). Accordingly, there was no correlation between "Length *O. cf. nigrivasis*" and "Day of the Year" (*r*=-0,067, *P*>0,05), as well as "Area *O. cf. nigrivasis*" and "Day of the Year" (*r*=-0,047, *P*>0,05), (Fig. 11).

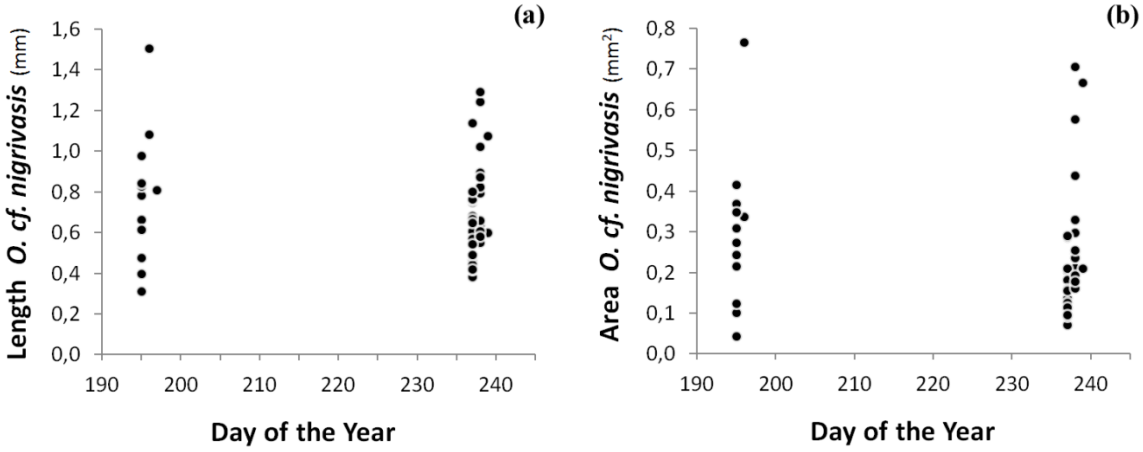

Fig. 11. Relation between: (a) the length of *O. cf. nigrivasis* and the day of the year; (b) the area of *O. cf. nigrivasis* and the day of the year.

Differences between sexes

Overall, the infection rate of males and females was similar (χ^2=4,413, *df*=2, *P*=0,111). There was no difference between the parasite burden (number of *O. cf. nigrivasis* per individual) of males and females (t_{24}=-0,059: *P*>0,05). Infected males had an average of 2,20 parasites per individual against 2,25 in each female. There were also no differences in the average number of *O. cf. nigrivasis* per individual between genders in July (t_4=-0,555: *P*>0,05) and in August (t_{17}=-0,215: *P*>0,05).

There were no significant differences between the dimensions of *O. cf. nigrivasis* parasiting male and female *X. laevis*: Area (t_{50}=-0,415: *P*>0,05). That tendency

remained when we analyzed only the individuals captured in July (n=13), (t_{11}=0,909: *P*>0,05), as well as in August (n=38; t_{11}=1,206: *P*>0,05).

Relation with frog SUL

No significant association between the number of *O. cf. nigrivasis* and the SUL of both male and female *X. laevis* was found (Males: *r*=-0,004, *P*>0,05; Females: *r*=0,05, *P*>0,05), (Fig. 12).

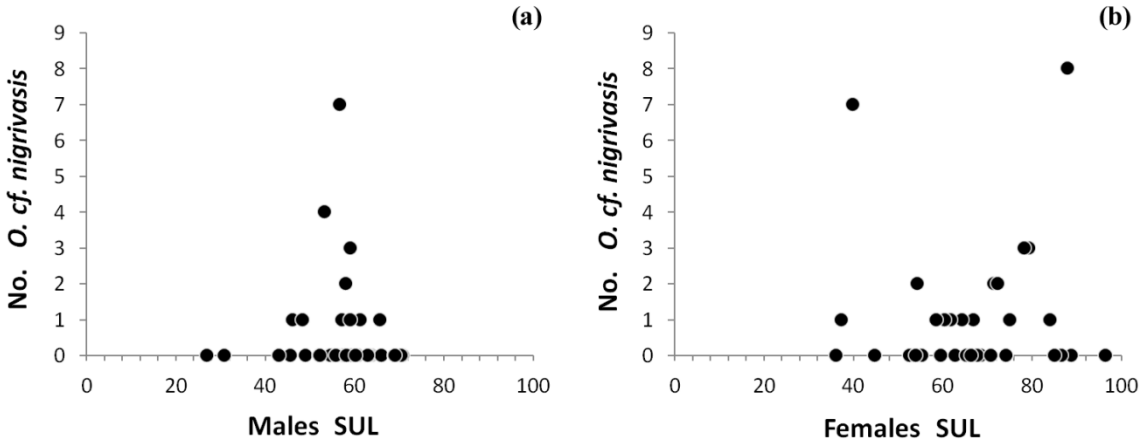

Fig. 12. Relation between: (a) the number of *O. cf. nigrivasis* and the SUL of male *X. laevis*; (b) the number of *O. cf. nigrivasis* and the SUL of female *X. laevis*.

There was a positive correlation between dimensions of *O. cf. nigrivasis* and SUL of female *X. laevis* (Length: *r*=0,417, *P*<0,05; Area: *r*=0,354, *P*<0,05). However, there were no significant relations for male *X. laevis* (Length: *r*=-0,051, *P*>0,05; Area: *r*=-0,090, *P*>0,05), (Fig. 13).

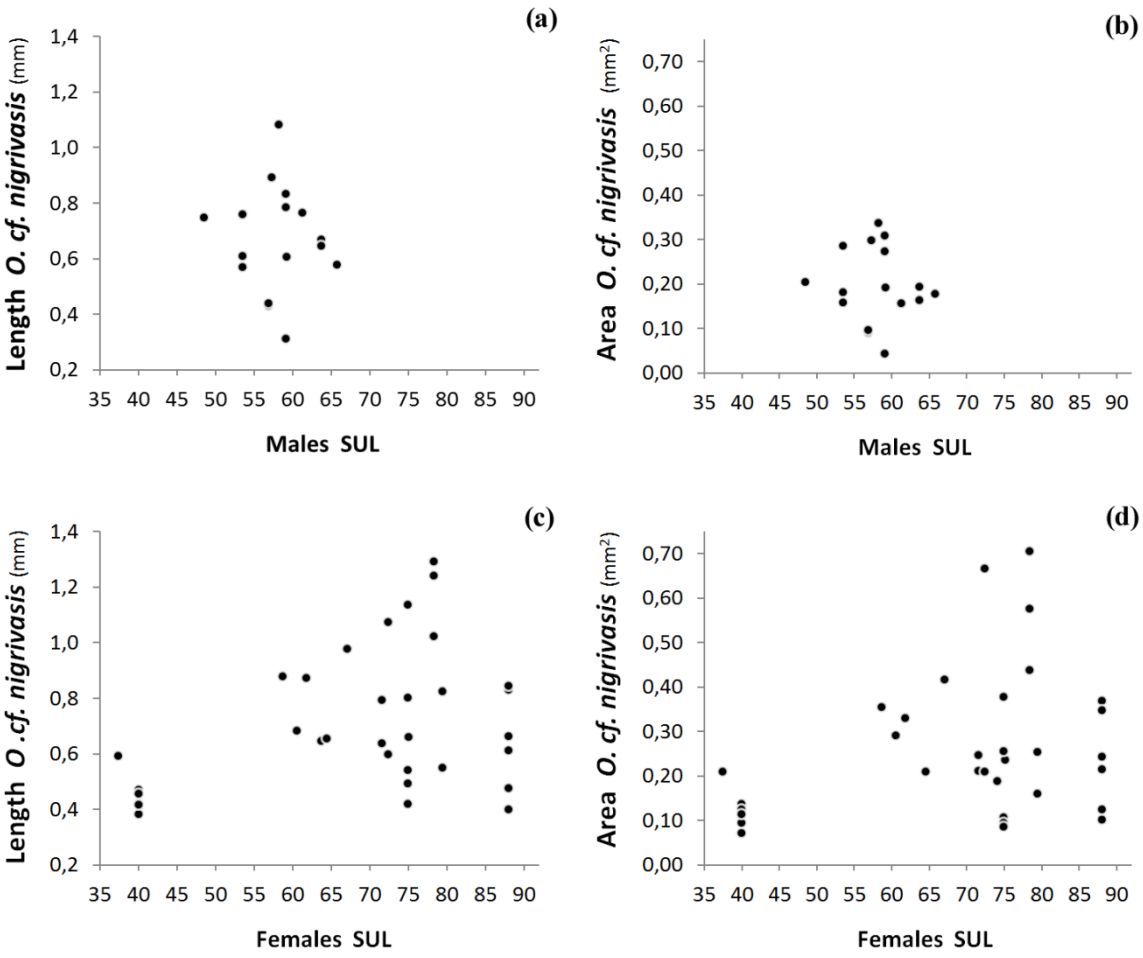

Fig. 13. Relation between: (a) the length of *O. cf. nigrivasis* and the SUL of male *X. laevis*; (b) the area of *O. cf. nigrivasis* and the SUL of male *X. laevis*; (c) the length of *O. cf. nigrivasis* and the SUL of female *X. laevis*; (d) the area of *O. cf. nigrivasis* and the SUL of female *X. laevis.*

All parasites

We found a total of 179 parasites in the dissected *X. laevis*. The prevalence of parasites in our sample was 69%, with a mean intensity of 3,25 parasites per host (55 of the 80 dissected frogs were infected with at least 1 parasite), (Table 4).

Table 4. Details of the helminth infection in *Xenopus laevis* in Oeiras (Portugal) in 2013.

Helminth species	Prevalence (%)	Abundance	Mean Intensity	Number recovered	Microhabitat
Protopolystoma xenopodis	55	1,43	2,59	114	Urinary bladder
Opisthodiscus cf. nigrivasis	33	0,73	2,23	58	Rectum
Unidentified A	9	0,09	1	7	Intestine
Total	68,75	2,24	3,25	179	

We estimated the probability of *X. laevis* be infected with *P. xenopodis* and *O. cf. nigrivasis* simultaneously. The value obtained – 18,15% of the sample, is quite approximated to the 20% that were observed.

Overall, prevalence was higher in August (83%) than in July (63%) and October (30%), as well as the mean intensity with values of 3,58 in August against 2,74 and 2,67 in July and in October, respectively.

There were no differences between the number of parasites found in July and August (t_{50}=-0,855: *P*>0,05).

The infection rate of males and females was similar (χ^2=2,258, *df*=3, *P*=0,521). There was no difference between the total number of parasites found in males and females (t_{53}=-0,130: *P*>0,05). Infected males had an average of 3,18 parasites per individual, while females had 3,30 each. There were also no differences when comparing the average number of parasites per individual between genders in July (t_{17}=-0,711: *P*>0,05) and in August (t_{31}=-0,214: *P*>0,05).

No significant association between the total number of parasites and the SUL of male and female *X. laevis* was found (Males: *r*=0,17, *P*>0,05; Females: *r*=0,06, *P*>0,05), (Fig. 14).

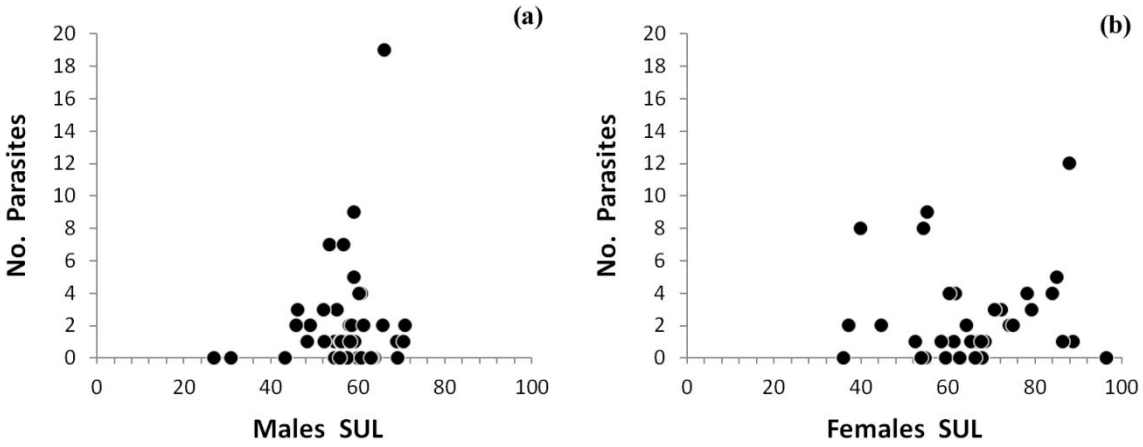

Fig. 14. Relation between: (a) the number of parasites and the SUL of male *X. laevis*; (b) the number of parasites and the SUL of female *X. laevis*.

All parasites

We found a total of 452 parasites in the 18 sampled *Pelophylax perezi*. All frogs were infected with at least 1 parasite species (prevalence 100%, mean intensity 25,11), (Table 5).

Table 5. Details of the parasite infection in *Pelophylax perezi* in Oeiras (Portugal) in 2014.

Helminth species	Prevalence (%)	Abundance	Mean Intensity	Number recovered	Microhabitat
Opisthodiscus cf. nigrivasis	50	2,33	4,67	42	Rectum
Sonsinotrema tacapense	61	16	26,27	289	Intestine
Unidentified B	56	4,28	7,7	77	Intestine
Rhabdias bufonis	50	0,78	1,56	14	Lungs
Unidentified C	33	1,56	4,67	28	Rectum
Total	100	25,11	25,11	452	

The infection rate of males and females was similar (χ^2=1,862, *df*=3, *P*=0,602). A single male was found to be infected with 153 parasites. Excluding this individual from the analysis, there was no difference between the total number of parasites found in males and females (t_{15}=0,568: *P*>0,05). Infected males had an average of 18,5 parasites per individual against 14,8 in each female.

Relation with frog SUL

There was a positive correlation between the total number of parasites and the SUL of male *P. perezi* (Males: *r*=0,62, *P*<0,05). This analysis was not possible for female *P. perezi* due to the small sample (Fig. 15).

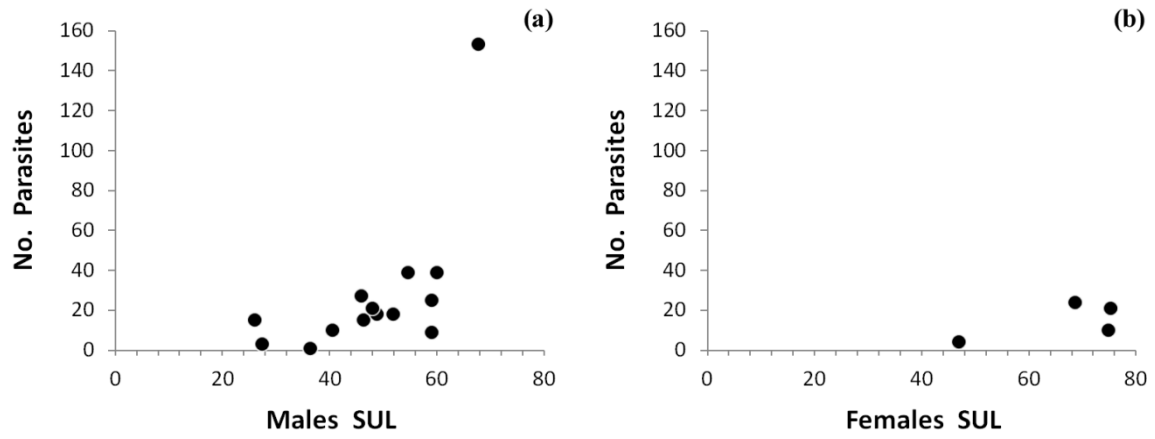

Fig. 15. Relation between: (a) the number of parasites and the SUL of male *P. perezi*; (b) the number of parasites and the SUL of female *P. perezi*.

COMPARISON OF HELMINTH BURDEN BETWEEN BOTH AMPHIBIANS

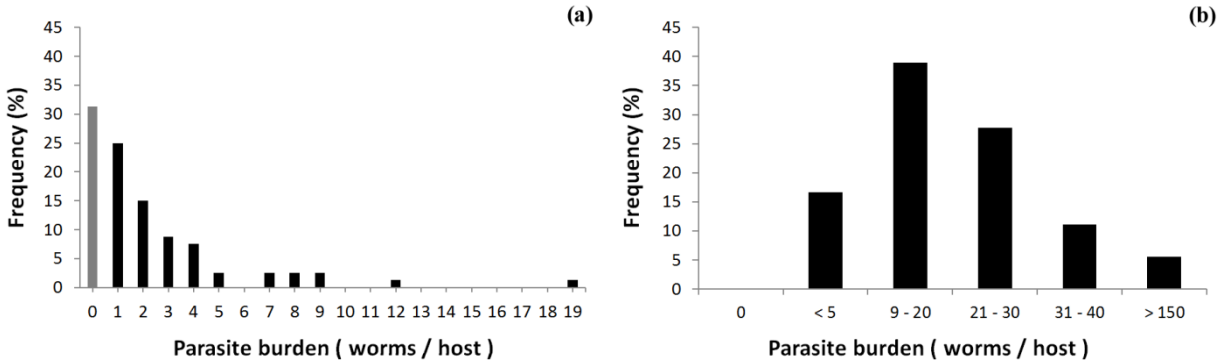

Fig. 16. Frequency distributions of parasite burden in: (a) *Xenopus laevis*; (b) *Pelophylax perezi*.

Approximately 82% of the infected *X. laevis* exhibited 1–4 parasites per host, with some cases ranging between 5 and 19 parasites per host (Fig. 16a).

Pelophylax perezi was mainly infected by 9–20 parasites (38,9%) and reached a common maximum of 40 worms per host. There were a few cases of individuals with less than 5 parasites (16,7%), as well as one case of a single individual infected with more than 150 parasites (Fig. 16b).

DISCUSSION

This was the first characterization of the parasite fauna of the invasive population of *Xenopus laevis* in Portugal. We found a parasite species native from Africa and documented for the first time individuals of *Opisthodiscus cf. nigrivasis* parasiting *X. laevis*. A third species, probably acquired after the introduction of *X. laevis* in these streams, remains unidentified. Also, we obtained data on how the biology of these helminths varies according to the characteristics of the hosts and to the season.

In this study, we found three helminth species in *Xenopus laevis*: *Protopolystoma xenopodis*, *Opisthodiscus cf. nigrivasis* and a third unidentified species. Of these, only *P. xenopodis* is currently known to parasitize *X. laevis*, whether in its native range or in the areas where it is invasive (Tinsley, 1996). This was an expected result, as the majority of the native parasites is lost when a host is introduced into a new environment (Torchin & Mitchell, 2004). Some species of parasites may not be present in the individuals of the introduced subpopulation, others may be brought together with their host but not meet the requirements to complete their life cycle in the new habitat, such as compatible intermediate hosts or a sufficient initial number of individuals to successfully maintain the effective size of the population.

Five species of helminths were found in *Pelophylax perezi*: *Opisthodiscus cf. nigrivasis*, *Sonsinotrema tacapense*, *Rhabdias bufonis* and two unidentified species. This value is far from the 44 species described for this frog (Roca *et al.*, 1984; Vojtkova & Roca, 1994; Vojtkova & Roca, 1996; Lluch *et al.*, 1985; Lluch et al., 1986a, 1986b, 1986c, 1986d; Navarro *et al.*, 1988; Navarro *et al.*, 1989; Navarro & Lluch, 1991; Navarro *et al.*, 1995; Navarro & Lluch, 2006). However, for each local population of *P. perezi*, helminth richness tends to be much lower: 16 species in the study of Navarro & Lluch (2006), and 9 species in the study of Lluch *et al.* (1986b). Even considering the lower richness of local populations, our results show that the helminth fauna of *P. perezi* at Barcarena may be still relatively impoverished. The *P. perezi* sample was relatively small and was collected mainly in a single season; both facts may contribute for the relatively low helminth richness.

Protopolystoma xenopodis had a prevalence of 55%, with an average of 2,59 parasites per infected host. According to Tinsley (1995), the majority of infected individuals carry only 1–2 adult *P. xenopodis*, despite the constant re-infection cycles and the longevity of 2,5 years of this parasite. Similar results were also found in our sample, where 70,5% of *X. laevis* were infected with one or two parasites, and 93% of the cases were comprised within the normal maximum of six adult parasites per host (Tinsley, 1996). There were however exceptional cases, as some individuals had 7, 9 and even 19 adult *P. xenopodis* in their bladders. Even with high levels of larval infection (mean 18 worms/host), such high values of adult *P. xenopodis* are rare, due to the high loss of parasites that occurs before maturation, leaving normally no more than six adult worms per host (Tinsley, 1996). Other authors found relatively high prevalence values to be the result of the high density of *X. laevis* individuals, which were densely aggregated in confined water bodies and experienced a high invasion rate of *P. xenopodis* (Tinsley & Jackson, 2002). The same reason may explain our results, but it is also possible that some frogs had a weak immune response to the damage caused by the juvenile stages of *P. xenopodis* in the kidneys, enabling a higher and easier rate of infection (R. C. Tinsley, pers. comm.; Tinsley & Jackson, 2002).

Additionally, it must be noted that for the above mentioned values, we only considered *P. xenopodis* that were found in the urinary bladder of *X. laevis*. The presence of *P. xenopodis* larvae in *X. laevis* kidneys was not assessed during this thesis, because it is a time consuming process and requires experience.

The second most abundant helminth that was found parasitizing *X. laevis* was *Opisthodiscus cf. nigrivasis*, with a prevalence of 33% and a mean intensity of 2,23 parasites per host, values that are very similar to those described for native frogs usually parasitized by this species, including *Pelophylax perezi* (Navarro & Lluch, 2006).

PARASITE AND HOST LIFE CYCLES

Protopolystoma xenopodis

In other populations, adult *P. xenopodis* are found in the urinary bladder of *X. laevis* and reproduce continuously throughout the life of the mature worm (mean 9 eggs/worm/day) (Jackson & Tinsley, 1998). Cross-fertilization seems to be the preferred choice amongst polystomes, however self-insemination probably occurs in

solitary individuals via the ovo-vitelline duct (Williams, 1960). The eggs are expelled into the external environment and infective oncomiracidia hatch in 22 days. Once contact has been established with a potential host the oncomiracidium enters the cloaca and migrates to the kidneys where it develops for approximately 2–3 months. It then migrates back to the urinary bladder where egg production begins 3–4 months post-infection (Tinsley, 2004; Tinsley & Jackson, 2002; Tinsley & Owen, 1975; Theunissen *et al.*, 2014).

In our study population there was no variation in the number of *P. xenopodis* per infected individual *X. laevis* throughout the months, and prevalence was similar in July and August, which may be explained by the constant cycles of re-infection of this parasite (Tinsley, 1995). The very low prevalence and high mean intensity of the October sample may be explained by the small number of individuals sampled in that month.

However, there was a decrease of the dimensions of *P. xenopodis* during the course of the sampling period. This may be explained by the features of *P. xenopodis* life cycle, being the result of the arrival of just-mature individuals, which probably infected the kidneys in spring and started to migrate to the urinary bladder in midsummer (August). Unfortunately the larval stages were not searched for, and so it is not possible to verify the variation of larval numbers in the kidneys.

The gender of the host does not seem to be a determining factor in the parasite's "first approach", since the rate of infection in males and females were similar. When infected, males and females tended to have similar numbers of parasites, contrary to what would be expected, since several authors (e.g. Poulin, 1996a; Navarro & Lluch, 2006) affirm that males of many animal species are more susceptible than females to parasite infections due to hormonal and biological factors.

However, *Protopolystoma xenopodis* present in male *X. laevis* were larger than in females. This could be due to recent infection of females by just-mature parasites proceeding from their kidneys. Female *X. laevis* invest highly in reproduction (McCoid & Frittz, 1989) and may become immune-depressed after egg-laying in spring and in summer. In spite of a possible recent infection, the amount of parasites was equivalent to those found in males, so indirectly it is possible that, all year round, males are a preferential target and/or infected with greater success by these monogeneans, as happens with other helminths (Poulin, 1996b).

We also found a positive relation of the length of *P. xenopodis* with the size of female *X. laevis*. Young (smaller) *X. laevis* may be more vulnerable to parasite infections, because they probably have not yet developed an immunological response towards these parasites (Tinsley & Jackson, 2002). Consequently, finding the largest *P. xenopodis* in the largest females may result from long-established host-parasite relations, as well as from the absence of signs of re-infection in older females, which possibly are immunologically more active (Tinsley & Jackson, 2002; Tinsley *et al.*, 2012). On the other hand, the area of these helminths decreased as the SUL of males of *X. laevis* increased.

The majority of *P. xenopodis* had between three and four inter-caecal anastomoses just as Tinsley & Jackson (1998) reported for *X. laevis* captured at the Cape region (South Africa), thought to be a genetic character strongly linked to parasite lineages (R. C. Tinsley, pers. comm.), pointing to the possibility that *X. laevis* introduced in Portugal could be from this region. The low variability may mean a low number of parasites and low genetic diversity in the initial stock of hosts (R. C. Tinsley, pers. comm.) introduced into Oeiras streams.

Opisthodiscus cf. nigrivasis

As for *P. xenopodis*, *O. cf. nigrivasis* doesn't seem to have any preference for infecting males or females. No differences were found in the number of *O. cf. nigrivasis* over the months. However the frogs sampled in August had a significantly higher prevalence, while in July there was a slightly higher mean intensity.

According to the life cycles documented for other parasites of the family Paramphistomidae, adult flukes in the rectum of frogs lay thin-shelled eggs which are deposited in the water with the feces. After hatching, the miracidia penetrates a snail and develop into cercariae in the tissues of the host during the next 90 days post infection. Then the cercariae are shed by the snails and encyst as metacercariae in the skin of tadpoles and adult amphibians. Normally, the infection occurs when the frogs ingest the sloughed epithelium bearing the metacercariae, with the excystment taking place in the rectum (Olsen, 1974; Baker, 2007). However, tadpoles, and possibly adults, can also become infected by ingesting free cercariae. In these cases, the cercariae encyst promptly in the mouth and pass to the rectum, where excystment takes place. These

flukes require 2 to 3 months to reach maturity and remain in the frogs for about a year (Olsen, 1974; Baker, 2007).

Previous studies of the diet of *X. laevis* showed a variation in the quantity of sloughed skin ingested over the months, being higher during the breeding season (Measey, 1998; Amaral & Rebelo, 2012). These variations may explain the different levels of prevalence and of mean intensity observed in different months, with a higher prevalence being found after the peak of reproductive activity, in August.

The absence of differences in the dimensions of *O. cf. nigrivasis* between July and August point to a non synchronization or decoupling of the parasite and the frog life cycles.

However, the dimensions of these helminths were positively related with the size of female *X. laevis*, while males presented no such relation. These values suggest a longer life or more abundant resources for these parasites in larger, therefore older, females.

COMPARISON OF THE HELMINTH FAUNA OF *X. LAEVIS* AND *P. PEREZI*

Sixty nine percent of the 80 sampled *X. laevis* were infected with at least one helminth, with a mean intensity of 3,25 parasites per host (in a total of 179 parasites). There were no differences between the number of parasites found in July and August; however the prevalence and the intensity of infection were slightly higher in August than in the other months, possibly due to the increase of just-mature parasites of at least one of the species. Also, no differences were found in the infection rates and in the number of parasites regarding the gender and the SUL of the host.

On the other hand, all the 18 individuals of *P. perezi* were infected with an average of 25 parasites per host (total of 452). As for *X. laevis*, there was no evidence that the gender of the host had an influence in the biology of these parasites. However, the number of parasites appeared to increase with the SUL of males of *P. perezi* (insufficient data for females).

Pelophylax perezi, as a native species, is naturally more parasitized (higher number of individuals of a higher number of species) than *Xenopus laevis*. This was expected (Dogiel, 1938 in Kuperman *et al.*, 2004; Torchin & Mitchell, 2004), due to the long period of co-existence of native amphibians and their parasites in these streams, as well as due to the absence of various species of parasites typical of *X. laevis*.

The only species of non-native parasite that managed to become established alongside with its host *X. laevis* was *P. xenopodis*, and the strict host specificity observed in anuran polystomes minimizes the chances of infecting the native frog (Theunissen, 2014). Thus, no evidence was found of transmission of parasites originating from Africa to the only species of frog whose habitat overlaps with the area of distribution of *X. laevis* in Portugal.

However, a reverse pattern of infection occurred, with the transmission of parasites only from *P. perezi* to *X. laevis*. This apparently happened for one of the species, while the third helminth is still unidentified, and may also proceed from *P. perezi*.

Common in *P. perezi*, *Opisthodiscus cf. nigrivasis* occurred in 50% of the sampled frogs, in which we observed well developed individuals. The same pattern was not found in *X. laevis*, in which these helminths were present but in smaller numbers and sizes (not shown). In the rectum of some individuals we found what appeared to be encysted metacercariae (not shown), suggesting that *X. laevis* may be being parasitized by ingesting larval stages of the parasite or an intermediate host carrier of them. Even without the levels of success shown when parasiting *P. perezi*, *O. cf. nigrivasis* seems to be using *X. laevis* as a vector or host. As nowadays these frogs have proliferated in certain areas of the streams, largely supplanting the numbers of *P. perezi* (R. Rebelo, pers. obs.), the overall population of *O. cf. nigrivasis* may actually depend more on *X. laevis* than on *P. perezi*.

Another three species of helminths were found in the digestive system of *P. perezi*, two of them with even higher values of prevalence and mean intensity than *O. cf. nigrivasis*. However, none of them were found parasitizing *X. laevis*, which could be an indication of a greater parasite-host specificity than that shown by *O. cf. nigrivasis*.

In conclusion, in addition to the known impacts that *Xenopus laevis* causes locally and around the world (e.g. Tinsley & McCoid, 1996; Lobos & Measey, 2002; Solis *et al.*, 2011; *Measey et al.*, 2012), it was suspected that it could carry parasites from Africa, which could be a menace to the activity and/or survival of native species, disturbing even more the balance of the ecosystem.

However this scenario was found to be not true in our study area, and in fact *X. laevis* was the species that was found to be infected by autochthonous parasites. Still, the parasite burden that they presented was not as high and wide as in the species they co-exist with, or as high as in the habitats where it is native, which could cause this species to be more able to dominate the environments where it was recently introduced.

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ANNEXES

Annex I. Coordinates of the visited sites (Latitude; Longitude; Decimal degrees).

	LATITUDE	LONGITUDE
LAGE stream		
Bairro Navegadores	38,730700	-9,318653
Jardins Marquês Pombal	38,692247	-9,315423
BARCARENA stream		
Ponte de S. Marcos	38,751701	-9,289837
Lugar do Bico	38,746022	-9,286857
Entre Lugar do Bico e Fábrica da Pólvora	38,744996	-9,286697
Fábrica da Pólvora	38,742023	-9,285632
Tributário/Foz do Tributário	38,743880	-9,287413
Viveiros	38,737758	-9,283427
Ribeira-Abaixo	38,735363	-9,282051
Murganhal	38,709142	-9,273126